Genetic background of dwarfism in the Friesian hors

Research goal: The gene responsible for dwarfism is located near the terminal end of the short arm of chromosome 14. Goal is to 1) finemap the region of association with newly recruited cases and controls, 2) develop and validate a DNA test and 3) analyze *FGFR4* as candidate gene.
Material and methods: DNA and RNA analysis of recently recruited dwarfs. Genotyping SNP markers on ECA14 using DNA sequencing. Selecting informative SNPs for RFLP assay and validate this DNA test in a larger group. *FGFR4*: characterizing exons and mutation detection using DNA and RNA sequence analysis.
Results: The region of association on chromosome 14 is bordered by BIEC2-238965 and BIEC2-239795. SNPs in between are associated with dwarfism, with BIEC2-239391 and BIEC2-239646 most significantly associated. It could not be confirmed that a mutation in *FGFR4* is represented in the coding sequence of the mRNA.
Conclusion: The region of association is bordered by BIEC2-238965 and BIEC2-239795. Some controls had the dwarfism genotype for all SNPs except BIEC2-239391 and BIEC2-239646. Therefore, BIEC2-239391 and BIEC2-239646 are the most interesting for further research. The SNP in *FGFR4* is unlikely to be a coding SNP. It is not well preserved and it could not be sequenced in cDNA. This could be due to the primers or steps in the protocols and therefore further investigation is needed to confirm this finding. It is also unlikely that there is an 18th exon in the horse. Next Generation sequencing will be performed on in a short period of time and its results should be used to identify and/or confirm candidate genes and mutations.

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Introduction

The Friesian horse originates from Friesland, the Netherlands. Near the end of the 19th century the KFPS (Royal Dutch Friesian Studbook, the Netherlands) was founded. Between 1900 and 1910 only sixteen foals were born each year[1]. After 1913 the number of individuals grew to around 400 (in 1976) and reached around 6500 horses in 2003. It is due to the early bottleneck that there is a loss of genetic diversity. It is also the reason why there is a high inbreeding coefficient of 14.0%[1]. This is partly due to the 'popular sire effect', because only 2.5% of all stallions are used for breeding[1].

Figure 1: *6 months old Friesian mare with dwarfism.Photograph courtesy of Roel Nijssen.*

In the Friesian horse breed several disorders have higher incidences than in other horse breeds[2]. Retained placenta's are for example only seen in 2-10% of foaling mares in other breeds, whereas this number is 54% in Friesian mares. Friesian horses are prone to insect bite hypersensitivity with a prevalence of 18% versus 8% in Shetland ponies[2]. Megaoesophagus is a condition which can be seen in Friesian horses of all ages. It is a presumed genetically determined neuromuscular disorder. Verrucous pastern dermopathy is also observed in Friesian horses and other coldblooded horses with long feathered fetlocks, though there is no proven genetic background. Hyperextension of the fetlock with poor hind limb propulsion and hyperrotation of the hind foot is seen more often in Friesian horses. Ruptures of the aortic arch, though uncommon, are also known in Friesian horses[2]. This disease is likely to be genetic because three cases were descendants from the same sire. Also, 57% of the horses admitted to Utrecht Equine Hospital with aortic ruptures were Friesian horses, whereas only 6% of the hospital populations are Friesians[3]. Due to different tendon properties tendon/ligament laxity can also be seen[2]. Given the relatively high incidence of these disorders in the Friesian horse it may be these disorders are related to a common feature which is or has been selected intensively[2].

A disorder seen in Friesian horses that is known to have a genetic background is dwarfism. Dwarfism is an abnormal development which in the Friesian horse causes disproportional growth (limbs and ribs do not grow to their full potential). If the normal bone remodeling process is disturbed, abnormal defects in the growth plates may arise, with dwarfism as an example. Although its complete etiology is not yet understood it may be caused by a local disturbance in a regulatory system for growth plate development. The incidence is 0.25% in the Friesian horse breed and affected animals show predominantly growth retardation in the ribs and limbs[4] . Figure 1 shows an affected Friesian foal.

Affected animals are approximately 25% smaller[2,4]. As mentioned, the head and back grow faster than limbs and ribs, emphasizing the disproportional growth even more. Bodyweight of affected animals is reduced by 50%. The thorax displays a dent around Th10-Th16 and the animals have a broad chest with a narrowing at the costochondral junction. Affected animals have long backs and short limbs. While front and hind limbs show hyperextension of fetlock joints and their gaits are abnormal due to flexor tendon laxity, the animals are capable of moving in every gait. Their hooves are long toed and narrow. The abdomen is rounded and

weak and poor muscle development causes the spinous processes to protrude. However, the animals have a normal appetite and coat and the reproductive organs are normal in sexually mature animals[4]. These findings combined cause a typical display of disproportional growth and phenotypically comparable forms of dwarfism are seen in dogs, humans and cattle[2,4, 5,6].

During pathologic examination abnormally short long bones and a distorted, S-shaped ribcage were found. The ribcage has enlarged and widened costochondral junctions (figure 2). It is distorted with an inward protrusion of the junction in the caudal part of the ribcage. Microscopy shows irregular cartilage-bone transition (figure 3). The metaphyseal growth zone is irregular and the hypertrophic zone width is increased. The chondrocyte columns are irregular and distended. Chondrocyte columns are thicker, and irregular bone-cartilage transitions can be found. In mature cases there can be slight retention of cartilage in the primary spongiosa[4]. Both the distal metacarpus, metatarsus and proximal first phalanx are limited in growth to the first 10 weeks. Bones where growth plates close last tend to grow faster. The first phalanx in mature dwarfs is broader though normal in length. Dysplasia of distal metaphysis of the metacarpus and metatarsus can be observed. Physeal growth retardation in limbs and ribs therefore cause the typical dwarf phenotype[4]. These results are comparable to findings in osteochondrodysplasia[7]. A recent study shows involvement of the GH-IGF1 axis is unlikely, although this cannot be ruled out[8].

Though the cause for dwarfism is unknown, it may be a local defect or disturbance in a regulatory system for growth plate development. It is an autosomal recessive monogenic trait in Friesian horses[4,9].

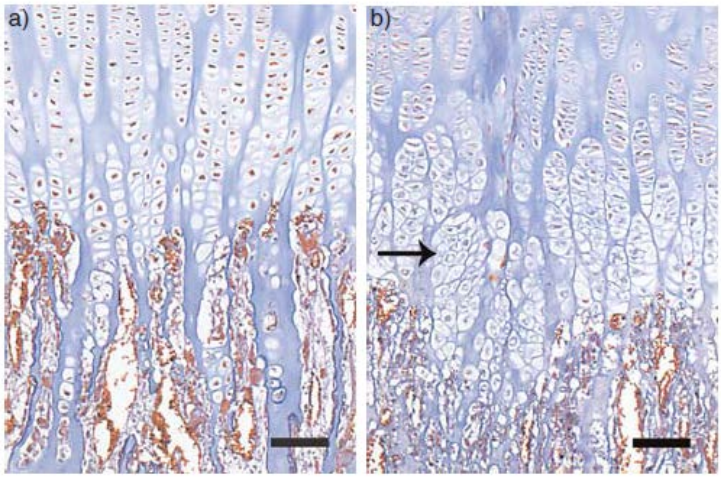

***Figure 2**: Microscopic view of normal (left) and affected (right) growth plates. A (left): costochondral junction of a 3-month-old healthy Friesian horse. B (right): costochondral junction of a 3 month-old dwarf; chondrocyte columns are disorganized and thicker than usual* [4]

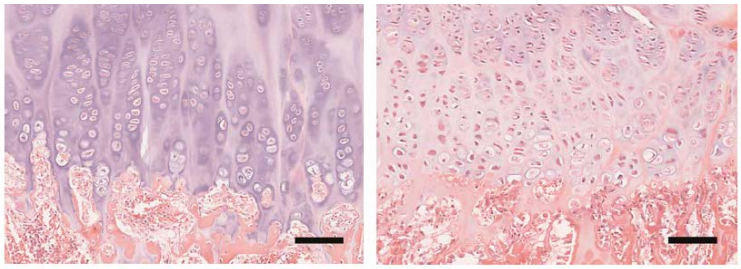

***Figure 3**: Microscopic view of normal (left) and affected (right) growth plates. Left: growth plate of a 2-year-old healthy Friesian horse. Right: same of a 2-year-old dwarf with disorganized chondrocytes (distal portion growth plate)* [4]

Genetic research

In 2010 Orr et. al. performed a genome wide association study (GWAS) with DNA material from ten dwarfs. A peak of association was found on chromosome 14, shown in figure 4A. BIEC2-239376 ($P = 4.54 \times 10^{-5}$) was the most significant and it is close to *PROP1*, a gene responsible for dwarfism caused by growth hormone deficiency in humans. The exons of *PROP1* were resequenced and no causative mutation was identified[7].

Further screening identified five other candidate genes: *ZNF346*, *COL23A1*, *B4GALT7*, *FGFR1* and *FGFR2*. *ZNF346* plays a role in apoptosis, important for the cartilage into bone transition. *COL23A1* has a role in collagen network formation, though specifics are unknown. *B4GALT7* also has a role in collagen network formation, and specifics are not known either. It has a role in connective tissue disorders and is related to disturbed fibril organization and proteoglycan synthesis. *FGFR1* and *FGFR2* mentioned as candidate genes. These genes have role in skeleton growth, mutations are known to cause skeletal dysplasia's and dwarfing syndromes. However, since *FGFR1* and *FGFR2* are not located on chromosome 14 it are highly unlikely candidate genes. Validation of these findings is needed[5,7].

Goal of this investigation is to first finemap the region of association previously mentioned with newly recruited cases, to validate a recently developed DNA test and to analyze a recently discovered SNP in *FGFR4* as candidate mutation and *FGFR4* as candidate gene.

Animals and methods

Animals

Six healthy horses and five cases were verified. Three control horses (two healthy horses, one case) were used for comparison. Of the healthy horses, one animal was 10 days old, another 24 months old. Two individuals were adult horses (3 years or older), though exact ages are unknown. Mean age of case animals was 327.6 days with a minimum of two days and a maximum of 4 years. RNA was isolated from liver tissue of an 18 months old euthanized KWPN mare.

Methods

DNA was isolated from blood and tissue using a DNA isolation kit. Fragments containing the SNPs (single nucleotide polymorphisms) were amplified using polymerase chain reaction (PCR). Temperatures in PCR Phusion protocols varied between 55°C and 63°C and a total reaction volume of 15uL, though relative amounts were used as supplier recommended. The DNA fragments were then used as a template in sequencing reactions using ABI Prism® BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems). Afterwards the DNA sequence was determined on a Genetic Analyzer 310 (Applied Biosystems) using capillary electrophoresis and automated detection of reaction products.

For genotyping of some SNPs the PCR products which were analyzed using DNA sequencing a SNaPshot multiplex kit (Applied Biosystems). One healthy individual and a carrier animal were used as controls. Protocols of the manufacturer (Applied Biosystems) were used and altered to optimize the results. For the SAP and ExoI treatment 7.5 uL PCR product, 2.5 ul SAP(1U/uL) and 0,5 uL ExoI (2U/uL) were incubated at 37°C for 60 minutes and 75°C for 20 minutes. A SNaPshot tercycling treatment was altered for better results. Another SAP treatment then followed, after which a SNaPshot reaction was performed and the products were analyzed on a Genetic Analyzer 310 (Applied Biosystems).

RNA was isolated from liver tissue of an 18 months old euthanized KWPN mare using an RNA isolation kit (Qiagen). cDNA was then synthesized from RNA. cDNA was then amplified using PCR and specific fragments were purified from gel, after which they underwent another PCR (semi-nested PCR). cDNA PCR product was then treated as described above and the DNA sequence was determined on a Genetic Analyzer 310 (Applied Biosystems) using capillary electrophoresis and detection of reaction products. Sequences were blasted on NCBI Blast: http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Results

DNA sequencing and SNaPshot results are summarized together with previously derived results by Manon Vos-Loohuis et. al. in appendix 1. There is one case heterozygous for all SNPs which is probably be due to a mix up of materials. One other case is heterozygous for BIEC2-239795. This horse is not a full bred Friesian horse but part Arabian. DNA from several newly recruited cases and controls are also shown in appendix one (dark blue and light yellow results). For BIEC2-238965, BIEC2-237718 and BIEC2-241575 some of the cases had a normal/healthy genotype (i.e. allele combination for SNP1 is GG). These SNPs (located on 2,99Mb and 7,69Mb) are therefore unlikely to be associated with dwarfism and are the borders of the region of association. SNPs located in between are more likely to be associated with dwarfism. Results for BIEC2-239391, *FGFR4*, BIEC2-239646, BIEC2-239889, BIEC2-240247 and BIEC2-240274 within expectation: all the cases are homozygous for the associated alleles and healthy individuals are either heterozygous or homozygous for the other allele. For BIEC2-239795 all the cases are identically homozygous. However, two control horses have the same genotype.

SNPnr	SNPname	Position (Mb)	P-value Chi2
2	237718	0.68	0.70053539
1	238965	2.99	1.55956E-17
7	239391	3.77	2.78015E-25
8	239646	4.23	1.44589E-23
9	239795	4.76	1.77882E-16
FGFR4	*FGFR4*	5.00	6.24832E-17
10	239889	5.11	--
6	240247	5.63	6.77193E-17
5	240274	5.72	4.68442E-13
4	241575	7.69	3.25483E-05

Table 1*: Chi2 results per SNP*

The SNP named *FGFR4* is located 70 basepairs upstream of the putative start codon of *FGFR4*. Since it was unknown whether this was a coding SNP or not, cDNA sequencing of *FGFR4* was performed. Blast comparison of the area of the mutation showed that it is a poorly conserved region. Apart from this, *FGFR4* in other species consists of 18 exons, however, the horse only has 17 exons. This 18th exon which did not seem present in the horse is located in front of the annotated first exon. Attempts to identify this exon in cDNA from a horse failed.

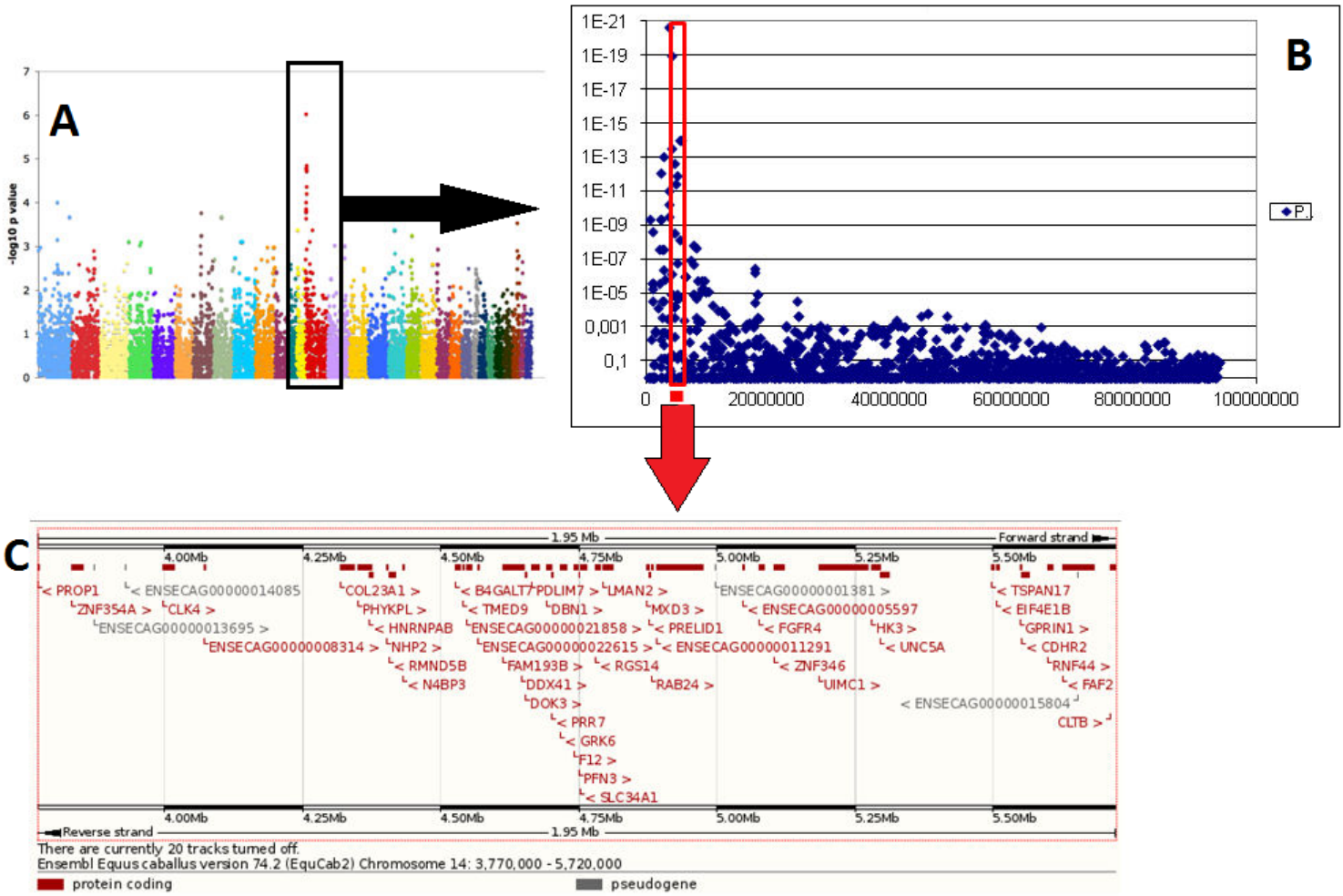

***Figure 4**: region of association on chromosome 14 (A and B by personal communication with Bart Ducro and Peter Leegwater) A) GWAS of dwarfism with an area of association on chromosome 14 (black box, red dots). B) Enlargement of the black box in figure A; chromosome 14 with a peak of association in the beginning of that chromosome. Red box and line: region where all dwarves had homozygous genotypes (3.77Mb-5.72Mb). C) Enlargement of the red box in figure B with genes located within that region (Ensembl.org).*

DNA test development

Another goal is to identify dwarfism carriers, horses which are heterozygous for the mutation associated with dwarfism in a DNA test. This test is developed in Wageningen by Ducro, Schurink et. al. They identified a region on chromosome 14 of 7.5Mb in size (6.87 Mb-8.18Mb) with 31 associated markers (figure 4B). Markers which are most associated are located around position 4 Mb. The most relevant markers are BIEC2-239391 (3.776.009 bp) and BIEC2-239493 (4.274.260 bp). The first SNP is also used in current investigation in Utrecht (SNPname: SNP7)[10].

If BIEC2-239391 and BIEC2-239493 are used for testing there is a 1.71% chance of a false-positive result. If two other SNPs are used, BIEC2-239646 and BIEC2-239709 are used, there is a 1.08% chance of getting a false-positive result. If all four are combined, all animals are identified correctly. BIEC2-239391 and BIEC2-239646 can be genotyped using a relatively cheap method; restriction fragment length polymorphism (RFLP) assay (figure 5). This has a 0,45% chance of a false-positive result. DNA sequencing using BIEC2-239709 can be added to reduce this chance to 0%, though this method is relatively expensive. BIEC2-239493 does not reduce this chance and therefore is not included in this test[10].

The protocol which needed to be validated:

1. Perform RFLP using BIEC2-239391 and BIEC2-239646
    a. Horse is identified as a non-carrier: no further steps are needed, the animal is not carrier of the mutation
    b. Horse is identified as carrier: perform PCR and DNA sequencing using BIEC2-239709.

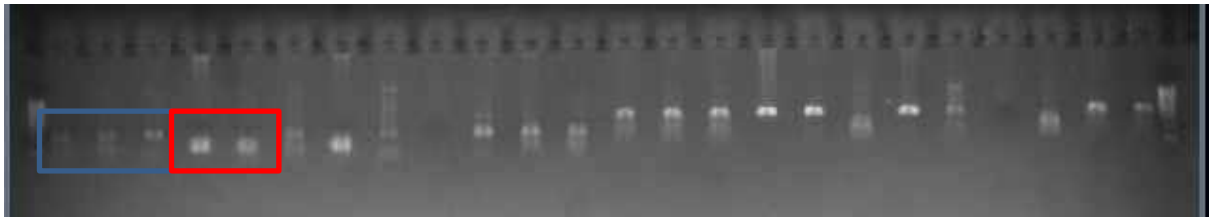

Figure 5*: RFLP assay (dwarfism) for BIEC2-239931 and BIEC2-239646 by Bart Ducro, personal communication. Heterozygous horses produce double bands. The first three animals (blue box) are examples of heterozygous animals (double bands). The next two slots (red box) contain PCR material of two dwarves (one low band). Homozygous control horses produce one upper band.*

Discussion

Dwarfism is known in a large number of animal species. Bovine Chondrodysplastic Dwarfism is a disorder in Japanese Brown Cattle probably caused by a mutation in the *LIMBIN* gene. There is no endochondral ossification of long bones and chondrocytes are irregularly arranged[11]. It is an autosomal recessive disorder and has most characteristics of chondrodysplasia, except for axial skeleton and facial structure degradation. Dexter cows are often heterozygous for a mutation in *ACAN*. It is a molecule in the cartilage matrix which causes a rather typical form of dwarfism; bulldogcalves[5].

In dogs several genes are associated with chondrodysplasia. Dogs with an *FGF4* retrogene are short-legged, like the dachshund. This form of dwarfism affects mostly long bones[12]. Oculoskeletal dysplasia is a form of chondrodysplasia in which the eyes are also affected. It is caused by a mutation in the *COL9A3* gene. Another mild and disproportionate form of dwarfism in Labrador retrievers is probably caused by a mutation in *COL11A2*[13]. A mutation in *SLC13A1* causes dwarfism in Miniature Poodles. Recently a mutation in *ITGA10* was reported for disproportionate dwarfism in the Norwegian Elkhound and the Karelian Bear dog[14].

In humans single mutations are known to cause dwarfism. Mutations in *POU1f1*, *GHRHR* or *PROP1* cause a proportional form of dwarfism with an autosomal recessive inheritance pattern[15]. Laron-type dwarfism is caused by growth hormone deficiency which is probably caused by a defect in the growth hormone receptor[6]. A *FGFR3* dominant activating mutation is seen in achondroplasia, thanatophoric dysplasia and hypochondroplasia, and is responsible for over 95% of achondroplasia cases, the most common type of human dwarfism[12]. Mice without *FGFR3* have an increase in skeletal growth because activation of *FGFR3* causes an inhibitory effect on the proliferation of chondrocytes. It also inhibits the differentiation of chondrocytes from the pre-hypertrophic zone to the hypertrophic zone. The mutation in this type of human dwarfism is a gain-of-function mutation, causing a considerably larger inhibitory effect[5].

This is just a brief overview of several mutations associated with dwarfism. DNA sequencing results of the SNPs found by Leegwater et. al. confirmed the original region associated with

dwarfism in the Friesian horse is located on chromosome 14. This region is bordered by BIEC2-238965 and BIEC2-241575. Genes located within this region (3.77Mb-5.72Mb) are shown in figure 4C and in appendix 2. As earlier results in appendix 1 show, this the case for all SNPs but BIEC2-239291 and BIEC239646. It could be that the other mutations are not expressed for 100% and that therefore healthy horses can carry alleles associated with dwarfism homozygously. The mutations could also be not 100% associated with dwarfism. These SNPs could also not be associated with dwarfism at all.

One candidate gene is *FGFR4*. Fibroblast growth factor receptors (FGFR) consist of a family of four single pass transmembrane receptors, each with tyrosine kinase activity. They are activated by fibroblast growth factors (FGF), which is a family of 15 polypeptide growth factors. *FGFR1*, *FGFR2* and *FGFR3* all undergo alternative splicing, but *FGFR4* does not[16]. While *FGFR1*, *2* and *3* exist of 19 exons in humans, *FGFR4* has only 18 exons in humans[17]. It has 17 exons in horses[18]. This additional exon is subject to alternative splicing. It also seems less conserved than the other three *FGFR*.[19] *FGFR4* binds to acidic and basic FGF (FGF-4, FGF-6, FGF-8 and FGF-9). It binds with less affinity to FGF-3 and FGF-5. At an early stage in normal mice FGFR4 is present in the gut, yolk sac and myotomal compartment of the somites. At a later stage it is present in developing skeletal muscles. Finally it is expressed in the ventricular zone and the cortex, and also in mammary carcinoma's. *FGFR4* is overall expressed relatively late in development of the lung. Its expression pattern overlaps with *FGFR3*[16]. In developing skeletal muscles, *FREK/FGFR4* is highly expressed. Inhibition of *FGFR4* leads to dramatic loss of limb muscles. This decrease varied between 10% and 100%[18].

Given the function of *FGFR4*, it may play an important but perhaps limited role in diseases of the muscle[19]. Mice lacking *FGFR4* developed normally. Their size was reduced by 10% at weaning. Mutant mice homozygous for *FGFR3* and *FGFR4* disruptions all displayed dwarfism by being 50% smaller at weaning. However, they are indistinguishable from normal mice at birth. They are largely infertile and appear sickly, most of them died after a few months[16].

FGFR3 mutations can result in diseases like achondroplasia, hypochondroplasia and thanatophoric dysplasia[20,21]. A Lys644Glu substitution in *FGFR3* causes dwarfism in mice. Homozygous mice are normal at birth but grew to become dwarfs. The mutation inhibits proliferation of growth plate chondrocytes. Usually, chondrocytes are divided into 4 types: resting, proliferating, maturing and hypertrophic chondrocytes. Mutants have very disorganized maturation zones and could not form long chondrocytes columns. The hypertrophic zone height was reduced and there was less expression of Ihh and collagen type II[21].

FGFR4 as a candidate gene is plausible when looking at sequencing data. All cases have both alleles GG and healthy horses are heterozygous AG. The *FGFR4* sequence from a variety of species was compared. While *FGFR4* exons are relatively well conserved (80-90% identical mostly), this is not the case for the region around the identified variation of the horse gene. This area, located upstream of the first exon is therefore not well conserved. Other animal species have 18 exons in *FGFR4*, the horse only 17. This additional exon in other animal species is located upstream of equine exon 1. cDNA analysis failed to identify this exon in the horse. This could be because it is indeed not present or because of unspecific primers or the used protocols.

In Friesian horses there is another developmental genetic disorder called hydrocephalus. It is disease which, though it is uncommon in horses, has a relatively high prevalence in Friesian horses[22]. The prevalence is 2,5 foals per 1000 births[2]. Where in humans only 40% of all hydrocephalus cases are suspected to be hereditary in origin, other publications suggested that this percentage is much higher in horses. However, he mode of transmission is unknown. In human babies the most common cause is a mutation in one of the genes encoding for *FGFR*. *FGFR* mutations are associated with not only hydrocephalus and chondrodysplasia, but also with dwarfism, as mentioned before. Personal communication with Bart Ducro revealed a region associated with hydrocephalus on chromosome 1. Since the region associated with hydrocephalus is located on chromosome 1 it can be excluded that both diseases are caused by a mutation in the same gene. These findings combined indicate that this SNP in *FGFR4* is unlikely to be a coding SNP.

The DNA test recently developed in Wageningen by Schurink and Ducro et. al. was successfully validated in a cohort of control and case animals. For some controls and cases the DNA material was of very poor quality, however, of the individuals that could be genotyped, all individuals could be genotyped according to known status as a carrier[10,23]. However, there is a chance that there are more haplotypes in the Friesian horse population than the investigated haplotypes. This could lead to false-positive or false-negative results when trying to identify a carrier[10].

Results of a Next Generation sequencing performed on six dwarves and six controls will soon come in. These results should be used for further identification of the causal mutation and thereby the causal gene. A list of possible candidate genes located in the finemapped region associated with dwarfism on chromosome 14 is given in appendix 2.

Conclusion

There is an area on chromosome 14 associated with dwarfism in the Friesian horse, located from 3,77Mb to 5,72Mb. This region is bordered by BIEC2-238965 and BIEC2-239795. Some controls had the dwarfism genotype for all SNPs except BIEC2-239391 and BIEC2-239646. Next Generation sequencing is performed during the first months of 2014 and its results should be used to validate earlier SNPs and to identify the causal SNP. The SNP in *FGFR4* is unlikely to be a coding SNP. It is poorly conserved and could not be sequenced from cDNA. A DNA test was recently successfully validated and will be used by the KFPS studbook[10].

Acknowledgement

I want to thank dr. P.A.J. Leegwater and dr. W. Back for their effort and supervising. Without their help I would not have been able to make this a successful project. I want to thank my daily supervisor, Manon Vos-Loohuis, for all her help when encountering practical problems.

I would also like to thank dr.ir. B.J. Ducro, B. Dibbits and dr.ir. A. Schurink from Wageningen for their help and time and including me in their research on developing a DNA test.

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Appendix 1: SNP results in associated region.

Status	2 = 237718		SQSTM1 3'UTR		1 = 238965		7 = 239391		8 = 239646		9 = 239795		SNP FGFr4		10 = 239889		6 = 240247		5 = 240274		4 = 241575		Old/new sample
control	A	A			G	G	A	A	G	G	G	G	C	C			C	C			G	G	old
control	A	A			G	G	A	A	G	G	G	G					C	C			G	G	old
control	A	C			G	G	A	A	G	G	G	A	C	G			C	A	C	T	G	G	old
control	A	A	A	A	G	G	A	A	G	G	G	G	C	C			C	C	C	C	G	G	old
control	A	C	G	G	G	G	A	A	G	G	G	A	C	G			C	A	C	T	G	G	old
control	C	C	G	G	G	T	A	A	G	G	G	G	C	C	T	C	C	C			G	G	old
control	C	C	G	A	G	G	A	A			G	G	C	C	T	T	C	C	C	C	G	G	old
control	A	A			G	T	A	A	G	G	G	G	C	C			C	C	C	C	G	G	old
control	A	C	G	A	G	G	A	A	G	G	G	A	C	G			C	A	C	T	G	G	old
control	A	A	A	A	G	G	A	A	G	G	G	A	C	G			C	A	C	T	G	G	old
control	C	C	G	A	G	G	A	A	G	G	G	G	C	C			C	C	C	C	G	G	old
control	A	C	G	A	G	G	A	A	G	G	G	A	C	G			C	A	C	T	G	G	old
control	A	C	G	G			A	A	G	G	G	A	C	G			C	A	C	T	G	G	old
control	A	C	G	G	G	G	A	A	G	G	G	A	C	G			C	A	C	T	G	G	old
control	A	C	G	G	G	T	A	A	G	G	G	A	C	G			C	A			G	G	old
control	A	C			G	G	A	A	G	G	G	G	C	C	T	T	C	C	C	C	G	G	old
control	A	C			G	T	A	G	G	A	G	A	C	G			C	A	C	T	G	A	old
control	A	C	G	A	G	G	A	A	G	G	G	G	C	C			C	C	C	C	G	G	old
control	A	A			G	G	A	A	G	G	G	G	C	C			C	C			G	G	old
control	A	A	G	A	G	G	A	A	G	G	G	G	C	C			C	C			G	G	old
control	C	C	G	G	G	G	A	A	G	G	G	A	C	G	T	C	C	A	C	T	G	G	old
control	C	C	G	A	G	G			G	G	G	G	C	C			C	C	C	C	G	G	old
control	C	C	G	A	G	G	A	A	G	G	G	G	C	C	T	T	C	C	C	C	G	G	old
control	C	C	A	A	G	G	A	A	G	G	G	G	C	C	T	C	C	C	C	C	G	G	old
control	A	C			G	G	A	A	G	G	G	A	C	G			C	A	C	T	G	G	old
control	A	C			G	G	A	A	G	G	G	A	C	G			C	A			G	G	old
control	A	C			G	G	A	A	G	A	G	A	C	C			C	C			G	A	old
control	A	A					A	A	G	G	G	G	C	C			C	C					old
control	A	C			G	G	A	A	G	G	G	G	C	C			C	C			G	G	old
control	A	C			G	G	A	A	G	G			C	G			C	A			G	G	old
control	C	C	G	A	G	G	A	A	G	A	G	A	C	C			C	C	C	C	G	A	old
control	C	C	G	A	G	G	A	G	G	G	G	A	G	G	C	C	A	A	T	T	G	A	old
control	A	C			G	T	A	G	G	A	G	A	C	G			C	A	C	T	G	G	old
control	C	C			G	G	A	A	G	G	G	G	C	C	T	C	C	C	C	C	G	A	old
control	A	C			G	T	A	G	G	A	G	A	C	G	T	C	C	A	C	T	G	A	old
control	C	C	G	G	T	T	A	G	G	A	G	A	C	G	C	C	C	A	C	T	G	A	old
control	A	C			G	T	A	G	G	A	G	A	C	G	T	C	C	A	C	T	G	A	old

control	A	C	G	G	T	T	A	G	G	A	G	A	C	G	C	C	C	A	C	T	G	A	old
control	A	C			G	T	A	G	G	A	A	A	G	G	C	C	A	A	T	T	G	A	old
control	C	C	G	A	G	G	A	G	G	A	G	A	C	G	T	C	A	A	T	T	G	A	old
control	A	C			G	G	A	G	G	A	G	A	C	G	T	C	C	A	C	T	G	A	old
control	A	C			G	T	A	G	G	A	G	A	C	G	T	C	C	A	C	T	A	A	old
control	C	C	G	G	G	G	A	G	G	G	G	G	C	G	T	C	C	A	C	T	G	A	old
control	C	C	G	G	G	G	A	A	G	G	G	A	C	G	T	C	C	A	C	T	G	G	old
control	A	C	G	G	T	T	A	G	G	A	A	A	G	G	C	C	A	A	T	T	G	A	old
control	A	C	G	A	G	T	A	G	G	A	G	A	C	G	T	C	C	A	C	T	G	G	old
control	A	C	G	G	T	T	A	G	G	A	G	A	C	G	T	C	C	A	C	T	A	A	old
Newcontrol					G	T	G	A	G	A	G	A			C	T	C	A	C	T	G	G	new
Newcontrol	A	C			G	T	G	A			G	A	C	G	C	T	C	A	C	T	G	G	new
Newcontrol	A	C			G	T	G	A	G	A	G	A	C	G	C	T			C	T	G	G	new
Newcontrol	C	C			G	G	A	A			A	A	C	G	C	T	C	A	C	T	G	A	new
Newcontrol	C	C			G	G	A	A			A	A	C	G	C	T	C	A	C	T	G	A	new
Newcontrol	C	C			G	G	G	A			G	G	C	G	C	T	C	A	C	T	G	A	new
case	A	A			T	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	G	A	old
case	C	C	G	G	T	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	G	A	old
case	A	A	G	G	T	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	A	A	old
case	A	A			T	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	G	G	old
case	A	A			T	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	G	A	old
case	A	C			G	T	A	G	G	A	G	A	C	G	T	C	C	A	C	T	G	G	old
case	C	C	G	G	T	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	G	G	old
case	C	C	G	G	G	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	G	A	old
case	A	A	G	G	T	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	G	G	old
case	A	C	G	G	T	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	G	A	old
case	C	C			G	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	G	A	old
case	A	A			T	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	G	A	old
case	C	C	G	G	T	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	G	A	old
case	C	C	G	G	T	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	A	A	old
case	A	A	G	G	T	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	G	A	old
case	C	C			T	T	G	G	A	A	G	A	G	G			A	A	T	T	G	G	old
case	C	C			T	T	G	G	A	A	A	A	G	G			A	A			G	A	old
case	A	A			T	T	G	G	A	A	A	A	G	G							G	G	old
case	C	C	G	G	G	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	A	A	old
case	A	A			T	T	G	G	A	A	A	A	G	G			A	A			G	A	old
case	C	C			T	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	A	A	old
case	A	C	G	G	G	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	A	A	old
case	C	C			T	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	G	A	old
case	A	A	G	G	T	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	G	A	old

case	C	C	G	G	G	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	A	A	old
case					T	T	G	G			A	A					A	A			G	A	old
case	A	A			T	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	G	G	old
case	C	C			G	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	A	A	old
case	C	C	G	G	T	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	G	A	old
case	C	A	G	G	T	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	G	G	old
case	C	C	G	G	T	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	G	A	old
newcase	C	C			T	T	G	G			A	A	G	G	C	C	A	A	T	T	G	G	new
newcase	A	A			T	T	G	G			A	A	G	G	C	C	A	A	T	T	G	A	new
newcase	A	C			G	T	G	G			A	A	G	G	C	C	A	A	T	T	A	A	new
newcase	C	C			G	G			A	A	A	A	G	G			A	A	T	T	A	A	new
newcase	A	A			T	T	G	G	A	A	A	A	G	G	C	C	A	A	T	T	G	A	new

Appendix 1*: SNP genotyping results. Results above red line are controls, results below red line are cases. SNPs are put in order of position. Dark green and light yellow: results of new cases and new controls. Light green and darker yellow: results of older cases and controls. SNP in orange and blue is a SNP in FGFR4. Grey and white gaps were left where no results were found. Preliminary results by Manon Vos-Loohuis and own results*

Ensembl Gene ID	Ensembl Transcript ID	Chromosome	Gene start (bp)	Gene end (bp)	Associated gene name	Description	Go Term Name (bp)	Go Term Name (cc)	Go Term Name (mf)
ENSECAG00000012431	ENSECAT00000012891	14	3832909	3852292	LOC100068186		regulation of transcription, DNA-dependent	intracellular	DNA binding | nucleic acid binding | zinc ion binding
ENSECAG00000014153	ENSECAT00000015128	14	3998237	4017579	LOC100058051		peptidyl-tyrosine phosphorylation	nucleus	ATP binding | protein kinase activity | protein serine/threonine kinase activity | protein tyrosine kinase activity
ENSECAG00000008314	ENSECAT00000008397	14	4070927	4074812			RNA-dependent DNA replication		RNA binding | RNA-directed DNA polymerase activity
ENSECAG00000008336	ENSECAT00000008711	14	4319016	4343909	LOC100068381				protein binding
ENSECAG00000013232	ENSECAT00000013914	14	4349953	4374301	AGXT2L2	alanine-glyoxylate aminotransferase 2-like 2 [Source:HGNC Symbol;Acc:28249]		mitochondrion	pyridoxal phosphate binding | transaminase activity
ENSECAG00000018242	ENSECAT00000019498	14	4371639	4376859					nucleic acid binding
ENSECAG00000022963	ENSECAT00000024585	14	4401153	4404791	LOC100058092				
ENSECAG00000000675	ENSECAT00000000710	14	4406050	4417967	LOC100058137				
ENSECAG00000009254	ENSECAT00000009470	14	4431340	4433790	LOC100058182			cytoplasm | membrane	
ENSECAG00000011175	ENSECAT00000011688	14	4526355	4535710	LOC100058225		fibril organization	Golgi apparatus | integral to membrane	galactosyltransferase activity | xylosylprotein 4-beta-galactosyltransferase activity
ENSECAG00000015165	ENSECAT00000016124	14	4539203	4542090	LOC100068443		transport	integral to membrane	
ENSECAG00000021858	ENSECAT00000023274	14	4547438	4556703			RNA-dependent DNA replication		RNA binding | RNA-directed DNA polymerase activity
ENSECAG00000022615	ENSECAT00000024278	14	4567542	4569558	LOC100068478		transport	ER-Golgi intermediate compartment | integral to membrane	
ENSECAG00000024626	ENSECAT00000026538	14	4613600	4643521	LOC100058268			extracellular region	hormone activity
ENSECAG00000009688	ENSECAT00000010318	14	4646268	4651341	LOC100058316				ATP binding | ATP-dependent helicase activity | helicase activity | nucleic acid binding | protein binding | zinc ion binding
ENSECAG00000022360	ENSECAT00000023830	14	4653461	4654908	DOK3	docking protein 3 [Source:HGNC Symbol;Acc:24583]	Ras protein signal transduction	cytoplasm	protein binding
ENSECAG00000022511	ENSECAT00000024189	14	4664360	4678793	LOC100058356		actin cytoskeleton	cytoplasm | ruffle |	protein binding | zinc ion

							organization	stress fiber	binding
ENSECAG00000010616	ENSECAT00000011230	14	4691349	4700723	DBN1		actin filament organization	actin cytoskeleton | intracellular	actin binding | protein binding
ENSECAG00000017561	ENSECAT00000018560	14	4701995	4703191	PRR7	proline rich 7 (synaptic) [Source:HGNC Symbol;Acc:28130]			
ENSECAG00000019815	ENSECAT00000021646	14	4716305	4727102	LOC100068570		protein amino acid phosphorylation		ATP binding | protein kinase activity | protein serine/threonine kinase activity | signal transducer activity
ENSECAG00000010619	ENSECAT00000011292	14	4742463	4749491	F12		blood coagulation	extracellular region | extracellular space	binding | calcium ion binding | catalytic activity | peptidase activity | serine-type endopeptidase activity
ENSECAG00000005555	ENSECAT00000005461	14	4750919	4751365	LOC100146754		cytoskeleton organization	actin cytoskeleton	actin binding
ENSECAG00000016737	ENSECAT00000018282	14	4753059	4764860	SLC34A1		bone remodeling	membrane | plasma membrane	sodium:dicarboxylate symporter activity | sodium-dependent phosphate transmembrane transporter activity
ENSECAG00000026999	ENSECAT00000029056	14	4780043	4788802	LOC100068605		mitosis	nucleus | spindle	signal transducer activity
ENSECAG00000012337	ENSECAT00000013137	14	4794548	4810740	LOC100058443			ER-Golgi intermediate compartment | membrane	
ENSECAG00000021155	ENSECAT00000022625	14	4873421	4877599	LOC100058486		negative regulation of transcription	nucleus	DNA binding | protein binding | transcription regulator activity
ENSECAG00000023460	ENSECAT00000025181	14	4878220	4880699	LOC100068620			mitochondrion	
ENSECAG00000024046	ENSECAT00000025913	14	4882098	4883944	LOC100058530		intracellular protein transport	intracellular | mitochondrion	GTP binding | GTPase activity | protein binding
ENSECAG00000011291	ENSECAT00000012000	14	4889831	4974316	NSD1		gastrulation with mouth forming second	nucleus	androgen receptor binding | calcium ion binding | chromatin binding | histone methyltransferase activity | histone-lysine N-methyltransferase activity | protein binding | transcription cofactor activity | zinc ion binding
ENSECAG00000005597	ENSECAT00000005496	14	5047962	5048945					

ENSECAG00000020869	ENSECAT00000023089	14	5077062	5084387	LOC100068679		fibroblast growth factor receptor signaling pathway	integral to membrane	ATP binding | fibroblast growth factor receptor activity | protein binding | protein kinase activity | protein serine/threonine kinase activity | protein tyrosine kinase activity
ENSECAG00000008904	ENSECAT00000009118	14	5103381	5121244	LOC100068705		apoptosis	intracellular | nucleolus	nucleic acid binding | protein binding | zinc ion binding
ENSECAG00000011567	ENSECAT00000012064	14	5184415	5272349	LOC100058611		DNA repair		DNA binding | retinoid X receptor binding
ENSECAG00000016508	ENSECAT00000017859	14	5279189	5295624	LOC100068725		carbohydrate metabolic process	mitochondrion	aspartic-type endopeptidase activity | ATP binding | hexokinase activity | phosphotransferase activity, alcohol group as acceptor
ENSECAG00000007408	ENSECAT00000008385	14	5296208	5311677	LOC100068742		signal transduction		protein binding
ENSECAG00000022322	ENSECAT00000023790	14	5496853	5501868	LOC100146751			integral to membrane	
ENSECAG00000022357	ENSECAT00000023982	14	5507086	5509820	LOC100068758		translational initiation	cytoplasm	RNA binding | translation initiation factor activity
ENSECAG00000002133	ENSECAT00000002039	14	5548206	5550681	GPRIN1	G protein regulated inducer of neurite outgrowth 1 [Source:HGNC Symbol;Acc:24835]	neuron projection development	plasma membrane	phosphoprotein binding
ENSECAG00000005816	ENSECAT00000005982	14	5552426	5565888	CDHR2		cell adhesion	integral to membrane | membrane | plasma membrane	calcium ion binding | protein binding
ENSECAG00000009095	ENSECAT00000009508	14	5599760	5608052	LOC100058652				protein binding | zinc ion binding
ENSECAG00000012970	ENSECAT00000013665	14	5626277	5682959	FAF2				protein binding
ENSECAG00000016628	ENSECAT00000017599	14	5711371	5729072	CLTB	clathrin, light chain (Lcb) [Source:HGNC Symbol;Acc:2091]	intracellular protein transport	clathrin coat of coated pit | clathrin coat of trans-Golgi network vesicle	protein binding | structural molecule activity

Appendix 2: Genes in associated region (genes within 3,77Mb-5.72Mb) Ensembl.org